Size-Exclusion HPLC of Protein Using a Narrow-Bore Column for Evaluation of Breadmaking Quality of Hard Spring Wheat Flours

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ABSTRACT

The objective of this study was to investigate whether a narrow-bore column (NBC) (300 × 4.5 mm, i.d.) improved analyses of unreduced proteins in flour by size-exclusion HPLC (SE-HPLC) and subsequent evaluation of breadmaking quality of hard spring wheat flours. Total protein extracts and SDS buffer extractable and unextractable proteins were analyzed by SE-HPLC. NBC separated proteins in 10 min at a flow rate of 0.5 mL/min with similar resolution to a regular column (300 × 7.8 mm, i.d.) which took 30 min. SE-HPLC absorbance area (AA) data obtained from an NBC showed comparable or superior repeatability and correlations with flour breadmaking characteristics when compared with those of a regular column. AA values of total protein that were calculated by adding AA values of SDS extractable and unextractable proteins showed greater repeatability and correlations with quality characteristics than those of actual total protein extracts. The improvements including employment of an NBC in SE-HPLC provide enhancement of rapid quality evaluation and decreased consumption of hazardous organic solvents.

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Molecular weight distribution of wheat proteins has been extensively analyzed by size-exclusion HPLC (SE-HPLC) since the initial work by Bietz (1984). Variations in molecular weight distribution of wheat proteins have been significantly associated with wheat end-use quality (Singh et al 1990a,b; Batey et al 1991; Gupta et al 1993; Bangur et al 1997; Huebner et al 1997; Bean et al 1998; Southan and MacRitchie 1999; Borneo and Khan 1999; Morel et al 2000; Zhu and Khan 2002; Suchy et al 2003, 2007; Kuktaitie et al 2004; Labuschagne et al 2004; Bekes et al 2006; Park et al 2006; Ohm et al 2006, 2008). Ohm et al (2008, 2009) found significant correlation coefficients between wheat quality characteristics and absorbance values of proteins separated by SE-HPLC at 0.05-min intervals. The results indicated that specific small protein fractions had distinct associations with quality characteristics. Ohm et al (2006, 2008, 2009) also reported that SE-HPLC data could be applied to develop robust prediction models of wheat quality characteristics by multivariate analysis methods which combined contributions of specific protein fractions to variations in quality characteristics. These results indicated that application of SE-HPLC data of protein should have high potential for the accurate and precise prediction of wheat quality characteristics.

However, the application of SE-HPLC for wheat quality evaluation has been difficult due to its complex and time-consuming procedure. Specifically, SE-HPLC is typically not rapid enough to analyze the large number of samples encountered in wheat breeding programs and industry. Larroque and Bekes (2000) introduced an SE-HPLC procedure that separated proteins more rapidly in 10 min rather than the standard 35 min of a regular procedure by employing a flow rate of 2 mL/min.

Recently, a narrow-bore column (NBC) with a 4.5 mm, i.d. has become commercially available for the analysis of wheat native proteins by SE-HPLC. The NBC usually operates at lower flow rates (0.10–0.50 mL/min) which result in increased sensitivity, reduced peak areas and decreased solvent consumption due to the narrower column i.d. versus a regular (larger bore) column (Neue 1997). The anticipated advantages of using an NBC are improved molecular weight distribution analysis of wheat proteins by shortening HPLC analysis time without loss of resolution as well as decreased consumption of hazardous organic solvents.

This research was performed to develop a rapid SE-HPLC procedure that can be employed for the quality evaluation of hard spring wheats. Specifically, the main objectives were to develop an SE-HPLC procedure using an NBC for the separation of flour native proteins and to test its potential for the rapid evaluation of breadmaking quality of hard spring wheat flours with decreased consumption of hazardous organic solvents.

MATERIALS AND METHODS

Thirty-three hard spring wheat cultivars grown at various locations were used in this study consisting of three cultivars grown at Brookings, SD; seven cultivars at Crookston, MN; 12 cultivars at Casselton, ND; and 11 cultivars at Minot, ND.

Wheat grains were cleaned in a Carter-Day Bulldog seed cleaner and scoured to remove beeswing and other extraneous matter. The scoured wheat was tempered to 16% moisture basis (mb) and conditioned for 16–18 hr. A Buhler experimental mill was used to mill-tempered wheats at an average feed rate of 175 g/min. Flours from three break (B1, B2, and B3) and two reduction (R1 and R2) sections were combined to patent flours and used for other analyses.

Flour nitrogen was determined by nitrogen combustion analysis according to Approved Method 46-30 (AACC International 2000) using a Dumas nitrogen analyzer (Leco Corp, St. Joseph, MI). Protein content was calculated as N × 5.7 (14% mb). Flour ash content was determined according to AACC Approved Method 08-01.

Flour mixing characteristics were analyzed by computerized farinograph (Brabender) with a 50-g bowl (AACC Approved Method 54-21). Farinograph mixing peak time was determined at optimum water absorption using software provided by Brabender.

Experimental breadbaking was performed at three wheat quality laboratories to evaluate flour breadmaking quality more accurately: Hard Spring and Durum Wheat Quality Laboratory, USDA-ARS, Fargo, ND; the Hard Spring Wheat Quality Laboratory, North Dakota State University, Fargo, ND; and the Hard Winter Wheat Quality Laboratory, USDA-ARS, Manhattan, KS. The optimized, pup-straight-dough AACC Approved Method 10-10B was used for experimental baking. Optimum baking water absorption and mix time were determined by the feel and appearance of the dough with referring to farinograph data. Bread loaf volume was determined by rapeseed displacement.

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Extraction and SE-HPLC of Proteins

Flour proteins were extracted as described by Gupta et al (1993) with minor modification (Ohm et al 2006). Total flour protein was extracted from a sample of 10 ± 0.05 mg (adjusted to 14% mb) by sonication (Sonic Dismembrator 100, Fisher Scientific) in extraction buffer. Flour samples were sonicated for 15 sec at the power setting of 10W output. Extraction buffer was 1 mL of 1% SDS and 0.1*M* sodium phosphate buffer (pH 6.9). The mixture was centrifuged for 15 min at 17,000 × *g* (Centrifuge 5424, Eppendorf AG, Hamburg, Germany) and the supernatant was filtered through a membrane filter (0.45 µm PVDF membrane, Sun Sri, Rockwood, TN). Immediately after filtering, the sample was heated for 2 min at 80°C and cooled at room temperature (Larroque et al 2000). SDS extractable and unextractable proteins were obtained according to the procedure of Gupta et al (1993). Flour (10 mg) was suspended in 1 mL of extraction buffer and stirred for 5 min at 2,000 rpm using a pulsing vortex mixer (Fisher Scientific). The mixture was centrifuged for 15 min at 17,000 × *g* and the extractable protein dissolved in supernatant was filtered and heated before SE-HPLC as described for total protein. The unextractable protein was obtained from the residue, using the same procedure for total protein extraction except for that a 30-sec sonication was used to disperse residues in solution as suggested by Gupta et al (1993).

SE-HPLC was performed using an Agilent 1100 Series (Agilent Technologies, Santa Clara, CA) using the procedure by Batey et al (1991). The HPLC procedure using a regular column was performed as a reference for comparison with the procedure using an NBC in this experiment. The total, extractable, and unextractable protein fractions were separated, respectively, by a regular column (300 × 7.8 mm, BIOSEP SEC S4000, Phenomenex, Torrance, CA) and an NBC (300 × 4.5 mm, BIOSEP SEC S4000, Phenomenex, Torrance, CA) with guard cartridges (BIOSEP SEC S4000, Phenomenex, Torrance, CA). Column packing material was hydrophilic bonded silica with particle size of 5 µm and pore size of 500 Å. Injection volume was 20 µL for the regular column (Gupta et al 1993). Injection volume for the NBC was 10 µL due to the smaller column volume than a regular column. Eluting solution was 50% acetonitrile in water with 0.1% (v/v) trifluroacetic acid and flow rate was 0.5 mL/min. Solutes were detected at 214 nm using Agilent 1200 photodiode array detector (Agilent Technologies, Waldbroann, Germany). These experiments were performed in triplicate and mean values were used for data analysis.

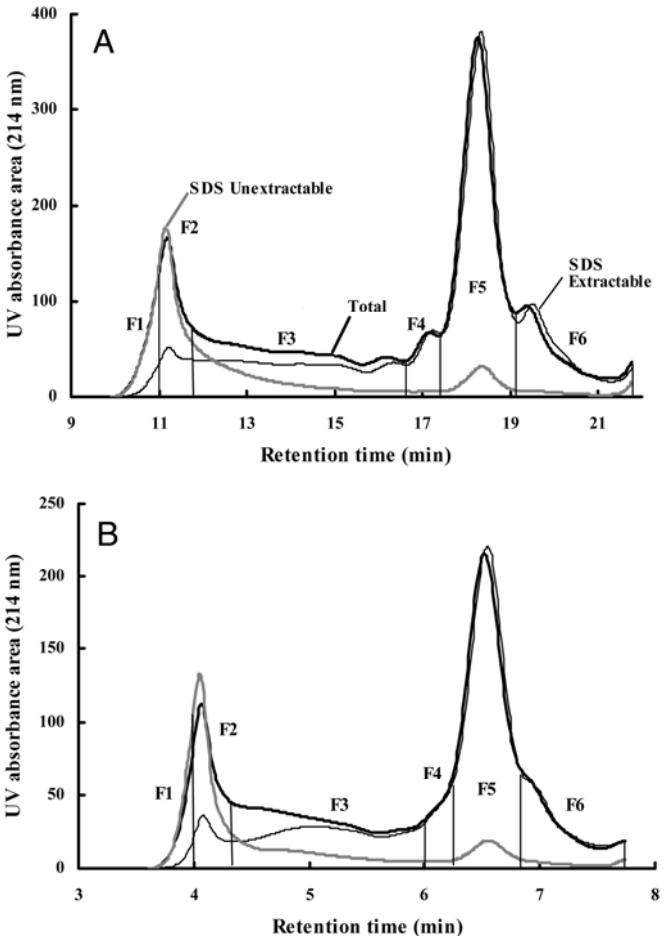

Fig. 1. Typical size-exclusion HPLC profiles of protein extracts separated on regular (**A**) and narrow bore columns (**B**).

SE-HPLC Data Analysis

Absorbance data from SE-HPLC chromatograms of protein extracts were interpolated and analyzed using an inhouse program that was developed using MATLAB (v.2008, The MathWorks, Natick, MA) (Ohm et al 2006, 2008, 2009). Absorbance values obtained from a regular column were interpolated to 0.01-min intervals by a spline method and were used to calculate absorbance area (AA). The AA was calculated by mean absorbance × time interval (0.01 min). The sum of AA for each retention time interval of 0.05 min between 9.9 and 20.5 min of runtime was used for data analysis. NBC absorbance data were interpolated to 0.002-min intervals by a spline method and the AA was calculated by mean absorbance × time interval of 0.002 min. The sum of AA for each retention time interval of 0.01 min between 3.6 and 7.7 min of runtime was used for data analysis. The AA values for total proteins were also mathematically estimated by adding AA values of extractable and unextractable protein fractions. The calculated AA values were then compared with those of actual total protein extracts which were obtained after sonication. Simple linear correlation coefficients (r) were calculated between both mean values of AA and quality parameters and shown as a continuous spectrum over retention time for each 0.05-min and 0.01-min retention interval of data obtained from the regular column and the NBC, respectively. The correlation spectrum obtained over individual retention time intervals, 0.05-min for a regular column and 0.01-min for an NBC, showed high enough resolution to identify protein fractions that had significant associations with quality characteristics. The AA values of major protein fractions (F1–F6 in Fig. 1) were also calculated. Retention time ranges of F1–F6 sections were determined based on their associations with quality characteristics found in previous researches by Morel et al (2000) and Ohm et al (2006, 2008, 2009).

Statistical Analyses

All of the HPLC experiments were performed in triplicate and statistical analyses were performed using SAS System for Windows (v.9.1, SAS Institute, Cary, NC). Mean and standard error values and r values were calculated using the PROC CORR procedure. Analysis of variance was performed using PROC GLM procedure. Relative standard deviations of AA values for all six fractions (F1–F6) (Fig. 1) were expressed as measures of repeatability of the SE-HPLC procedure.

RESULTS AND DISCUSSION

The sample set of hard spring wheat flours showed protein content and mixing and baking characteristics within the ranges expected of typical hard red spring wheat flours (Table I) (Maghirang et al 2006). The mean farinograph peak time showed a lower value than that reported by Maghirang et al (2006) probably due to the difference of sizes of farinograph mixing bowls and other settings. The 50-g mixing bowl was employed in this experiment while Maghirang et al (2006) used 10 g of flour to obtain farinograph parameters.

Comparison of HPLC Profiles

The mean SE-HPLC elution profiles of each protein extract are shown in Fig. 1. Total elution time was 21.5 min for the regular column (Fig. 1A) and 7.8 min for the NBC (Fig. 1B), showing similar protein elution profiles. The small peaks that were eluted at F4 and F6 showed lower resolution for the NBC when compared with those of the regular column. Larroque and Bekes (2000) also reported similar elution profiles when proteins were separated by a regular column at a flow rate 2 mL/min. Rapid SE-HPLC of a large sample set is seriously limited by the time required for sample preparation including weighing, extraction of proteins, etc., and HPLC runs (Larroque and Bekes 2000). The AA values of total protein extracts were also estimated by adding AA values of extractable proteins and unextractable proteins to save time required for sample preparation and HPLC analysis. The calculated AA values were larger than those of actual protein extracts as indicated in Fig. 1. This observation is probably due to the more extensive procedures for sequential extractions of SDS soluble and insoluble proteins than total proteins in this experiment (Morel et al 2000).

Analyses of variances were performed for AA values of six fractions of SE-HPLC chromatograms among flour samples (Fig. 1). When SE-HPLC of protein extract was performed using a regular column, F values from analysis of variance (Table II) of the protein fraction were not significant ($P < 0.05$) for all AA values. AA values for F2 and F4 of the total extract and F3 and F4 of SDS unextractable protein did not show significant variation among flours. When the NBC was used, F values were significant ($P < 0.05$) for all AA values except for F4 from the unextractable protein, which has no practical significance due to its low association with quality characteristics (Fig. 1). F values of the NBC were generally comparable or superior to those of the regular column. This result indicated that the use of the NBC improved the estimation of variances of the AA values among flour samples, which was likely due to a more stable separation of proteins with the reduced errors in an SE-HPLC run.

Repeatability of SE-HPLC was expressed using the relative standard deviation of the AA value (Table II), where the larger values indicate lower repeatability of the SE-HPLC method. Use of the NBC showed higher repeatability for AA values for F2 and F4 of the total protein extract. No pronounced difference in repeatability was observed for AA values of the other protein fractions. This result indicated that more reproducible results were attained when SE-HPLC was performed using an NBC despite the shorter analysis time. When compared with actual total protein extracts, the calculated AA values showed more significant F values and higher repeatability (Table II). These results indicated that variations of AA values among flour samples might be estimated more precisely from the calculated AA values and with fewer errors involved with HPLC analysis.

Significant ($P < 0.001$) correlations occurred between AA values of the regular column and the NBC for each chromatogram fraction (Table III). These results indicated that variations of AA values obtained from the NBC were similar to those from the regular column among flour samples and column × flour interaction was not significant. While AA values for F3 and F6 of the total protein extracts showed low correlations between the regular column and the NBC, the calculated AA values indicated greater correlation between them. This result indicated that calculated AA values might be used to estimate the variation of AA values of total proteins in this study.

Relationships of SE-HPLC Total Area and Flour Protein Content

Total SE-HPLC areas of total protein extracts represent the quantity of proteins in flour. There were highly significant correlations between flour protein contents and individual total SE-HPLC areas obtained using a regular column and an NBC (Fig. 2). Very high R^2 values for both a regular column and an NBC indi-

TABLE I
Mean and Standard Error Values of Flour Quality Characteristics

Quality Characteristics	Mean	Standard Error
Flour protein (%, 14% mb)	12.8	1.3
Flour ash (%, 14% mb)	0.66	0.06
Farinograph peak time (min)	5.9	1.4
Baking water absorption (%, 14% mb)	64.1	2.7
Bread loaf volume (cm^3)	861	94

TABLE II
***F*-Values Among Flour Samples and Repeatability for Absorbance Areas of SE-HPLC Fractions Attained on a Regular and a Narrow Bore Column (NBC)**

	F Value[a]		Repeatability (%)	
Protein Extracts[b]	Regular	NBC	Regular	NBC
Total				
F1	6.4***	3.0***	13.2	23.6
F2	4.6***	6.1***	10.1	9.4
F3	1.1ns	1.6*	20.6	14.1
F4	6.9***	30.0***	12.8	6.1
F5	21.7***	72.4***	4.5	2.7
F6	1.6ns	3.3***	19.0	8.2
TA	4.3***	14.7***	9.3	4.9
Total calculated				
F1	9.2***	6.1***	8.3	10.5
F2	17.9***	10.9***	5.1	6.5
F3	19.9***	18.8***	4.3	4.5
F4	127.1***	147.5***	3.1	2.8
F5	175.9***	197.7***	1.6	1.7
F6	14.9***	18.4***	3.4	3.8
TA	128.2***	75.2***	1.7	2.2
Extractable				
F1	12.7***	11.4***	8.8	10.7
F2	22.8***	10.9***	5.4	8.0
F3	17.0***	29.5***	5.2	3.9
F4	59.7***	39.9***	4.7	5.6
F5	23.0***	23.0***	4.4	4.9
F6	10.7***	13.9***	4.3	4.8
TA	22.7***	21.9***	4.2	4.3
Unextractable				
F1	8.8***	6.1***	9.4	11.8
F2	11.2***	10.9***	6.8	6.9
F3	2.7***	2.2**	14.0	16.6
F4	1.6ns	1.9*	38.3	30.9
F5	1.5ns	1.7*	52.5	50.5
F6	1.2ns	1.4ns	28.4	28.2
TA	4.4***	3.8***	11.9	12.7

[a] *,**,***, F value is significant at $P < 0.05$, 0.01, and 0.001, respectively; ns, not significant at $P < 0.05$.
[b] F1–F6, protein fractions separated by size-exclusion HPLC; TA, total absorbance area of a protein extract.

TABLE III
Correlation Coefficients Between Absorbance Areas of SE-HPLC Fractions Attained on a Regular and a Narrow Bore Column[a]

		Protein Extracts		
SE-HPLC Fractions[b]	Total Extracts	Total Calculated	Extractable	Unextractable
F1	0.981	0.940	0.930	0.945
F2	0.966	0.947	0.981	0.973
F3	0.768	0.995	0.996	0.994
F4	0.936	0.993	0.993	0.990
F5	0.978	0.993	0.994	0.984
F6	0.594	0.859	0.837	0.838
Total area	0.906	0.997	0.996	0.995

[a] All correlation coefficients are significant at $P < 0.001$.
[b] F1–F6 protein fractions separated by size-exclusion HPLC.

cated that total AA values obtained using an NBC could be employed to estimate protein contents as well as a regular column. There were significant correlation coefficients between flour protein content and SE-HPLC absorbance areas of total protein obtained from the NBC (Fig. 3). Flour protein content had higher correlations with absorbance area values of actual protein extracts at F1, F2, F4, and F5 than F3 and F6 (Fig. 3A). This result suggests that quantitative variation of flour protein was affected mainly by high molecular weight polymeric proteins and monomeric gliadin protein fractions. The total areas, which were calculated from data of SDS extractable and unextractable proteins showed greater R^2 values than those obtained from actual total protein extracts (Fig. 2). Absorbance area data which were calculated from data of SDS extractable and unextractable proteins showed high correlations for protein fractions eluted at F3 as well as other fractions except for F6 (Fig. 3B). These results were most likely due to the larger values of calculated AA values that represented quantitative variations of flour protein more accurately than those of actual extracts. Absorbance area data which were obtained using a regular column also showed similar results (data not shown).

Relationships of SE-HPLC Absorbance Areas to Breadmaking Quality

The relationship of SE-HPLC profiles to baking quality characteristics was investigated by calculating *r* values with AA values over retention time (Ohm et al 2008, 2009). SDS extractable proteins eluted at F4 and F5 were significantly ($P < 0.01$) correlated with farinograph water absorptions (Fig. 4). Specifically, proteins eluted at F4 showed greater correlations with water absorption than other protein fractions. Although HPLC profiles obtained from the NBC showed lower resolution of proteins eluted at F4 section than the regular column (Fig. 1), there was no pronounced difference between the correlation profiles of the two columns (Fig. 4). While flour protein content had an *r* value of 0.60 with water absorption, AA values showed a maximum *r* value of 0.77 for the regular column and 0.76 for the NBC (Fig. 4). Ohm et al (2006) also reported significant associations between F4 AA values and mixograph water absorption in hard winter wheat flours.

SDS unextractable polymeric proteins have a strong effect on dough strength parameters due to the greater associations between high molecular weight glutenin subunits (Gupta et al 1993). Bean et al (1998) reported that nitrogen content of SDS unextractable

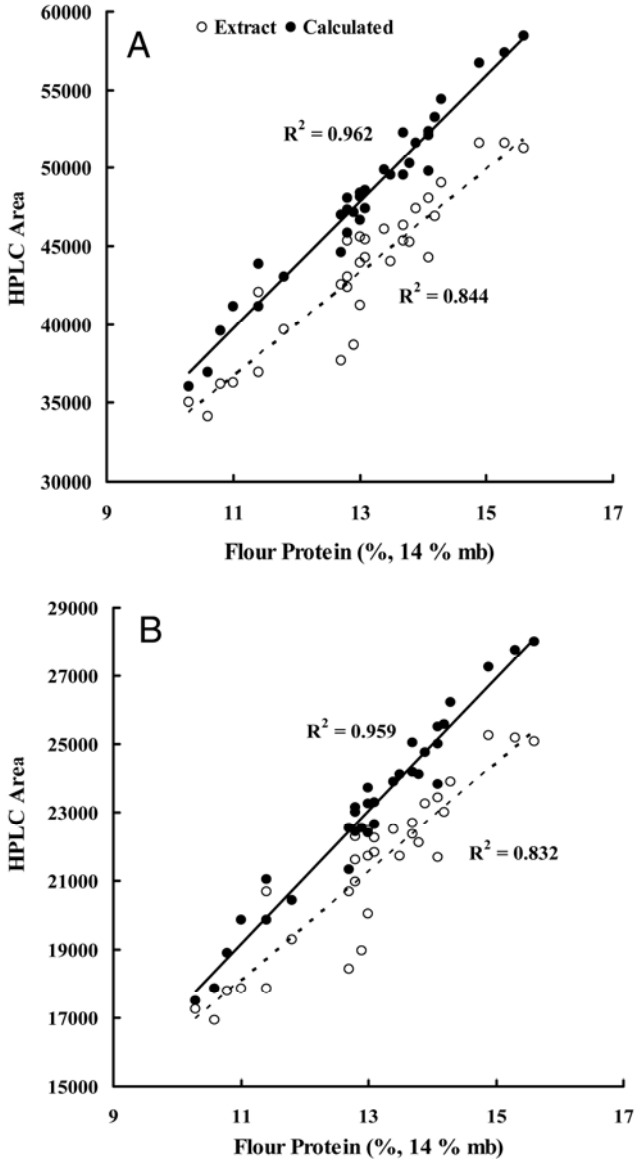

Fig. 2. Relationships between flour protein content and size-exclusion HPLC total absorbance areas obtained from total protein extracts (**A**) and calculated from data of SDS extractable and unextractable proteins (**B**).

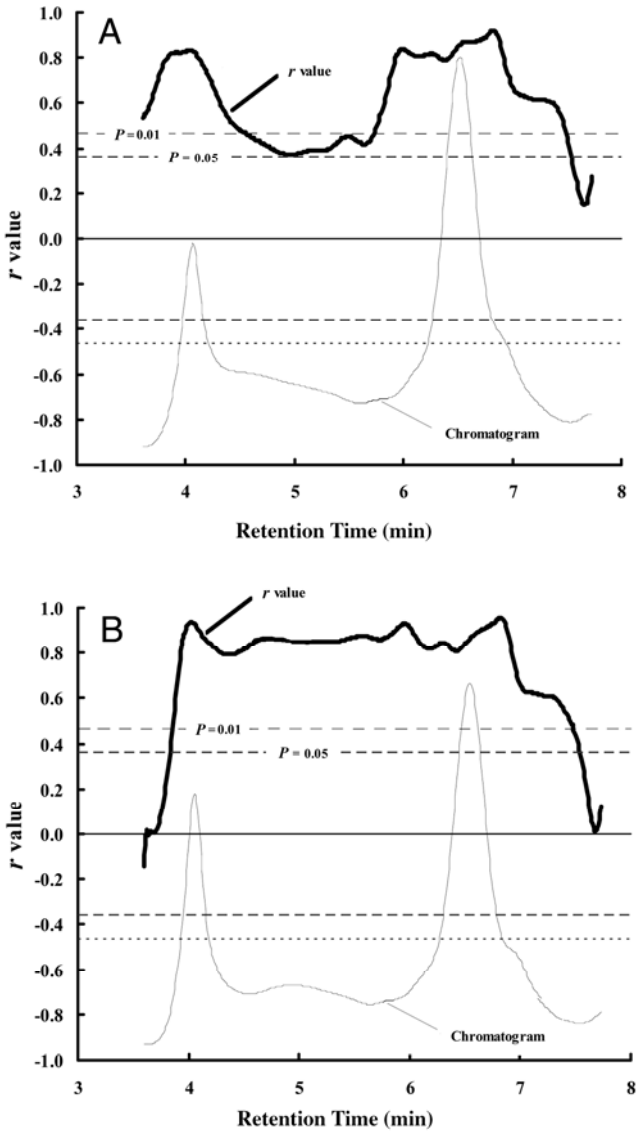

Fig. 3. Correlations (*r*) between flour protein contents and size-exclusion HPLC absorbance areas of total protein obtained from actual extracts (**A**) and calculated from data of SDS extractable and unextractable proteins (**B**).

flour pellet had significant correlations with dough strength parameters, indicating that unextractable polymeric proteins in pellet were responsible for the correlation. Correlation coefficients that were obtained from AA values of SDS unextractable proteins and farinograph peak times are shown in Fig. 5. The correlation spectrum indicated that unextractable polymeric proteins significantly affected farinograph peak times as reported by other researchers (Gupta et al 1993; Bean et al 1998). The correlation spectrum specifically indicated that variation of farinograph peak time was greatly affected by high molecular weight polymeric proteins of SDS unextractable proteins eluted at F1 and F2 (Fig. 5). Ohm et al (2006, 2008, 2009) also reported that the high molecular weight polymeric protein fraction had greater correlations with mixograph peak time and tolerance than other polymeric proteins. There was no difference in correlation profiles between the regular column and the NBC. Absorbance area values of F1 and F2 obtained from the NBC showed slightly lower *r* values with farinograph peak times than those of the regular column. However, the differences were not considered great enough to affect evaluation of flour quality because the *r* values were significant at $P < 0.01$ for both columns (Fig. 5B).

Correlation spectrums indicated that bread loaf volumes had significant associations with AA values of total protein extracts obtained from both columns (Fig. 6). Calculated AA values that were obtained by adding AA values of SDS extractable and unextractable proteins on corresponding retention time intervals (Fig. 1) showed greater *r* values with bread volume than those of actual protein extracts (Fig. 6B and C). Calculated AA values at F3 were significantly correlated with bread loaf volumes (Fig. 6B and D) while those of actual protein extracts had low or insignificant ($P > 0.05$) *r* values (Fig. 6A and C) probably due to the larger quantitative variations of calculated value as previously noted.

Bread loaf volume was greatly affected by quantitative variation of proteins as indicated by a significant correlation with flour protein content ($r = 0.84$, $P < 0.01$) in this study. Protein fractions eluted at F1, F2, F4, and F6 (Fig. 1) showed greater *r* values with loaf volume than other fractions from total protein extracts (Fig. 5). Those protein fractions also showed significant *r* values with water absorptions and mixing peak times. These results indicated that specific protein fractions affected variation in breadmaking characteristics more distinctively than other protein fractions in this sample set. Ohm et al (2006, 2008, 2009) also reported the

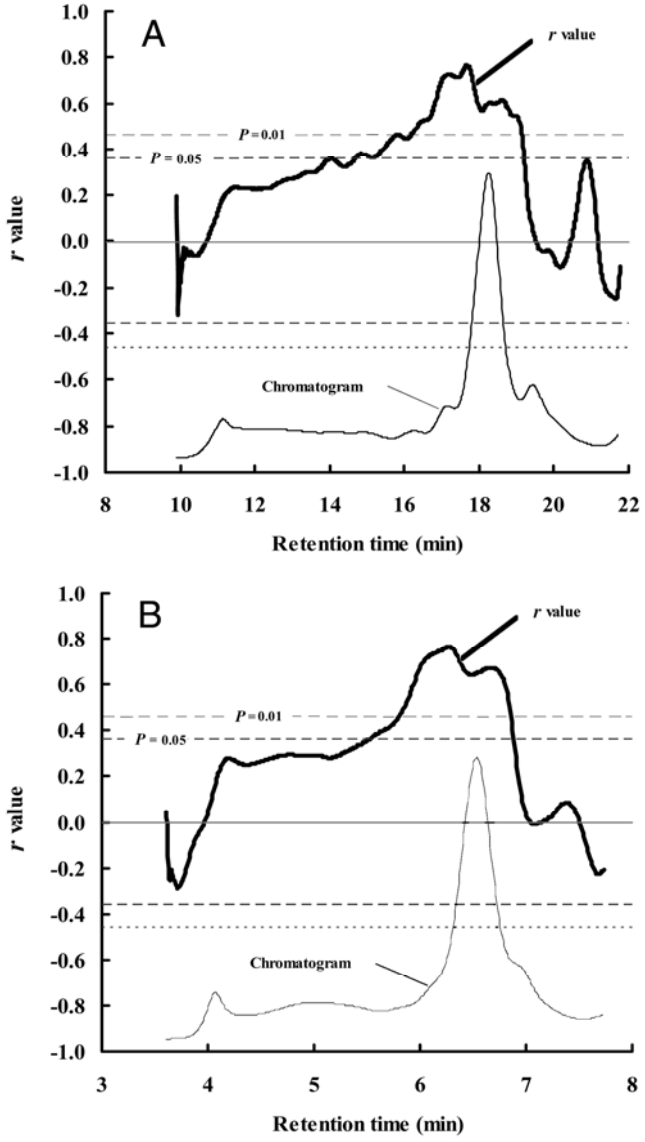

Fig. 4. Correlations (*r*) between farinograph water absorption (%, 14% mb) and absorbance areas of SDS extractable proteins obtained from a size-exclusion HPLC using a regular column (**A**) and a narrow bore column (**B**).

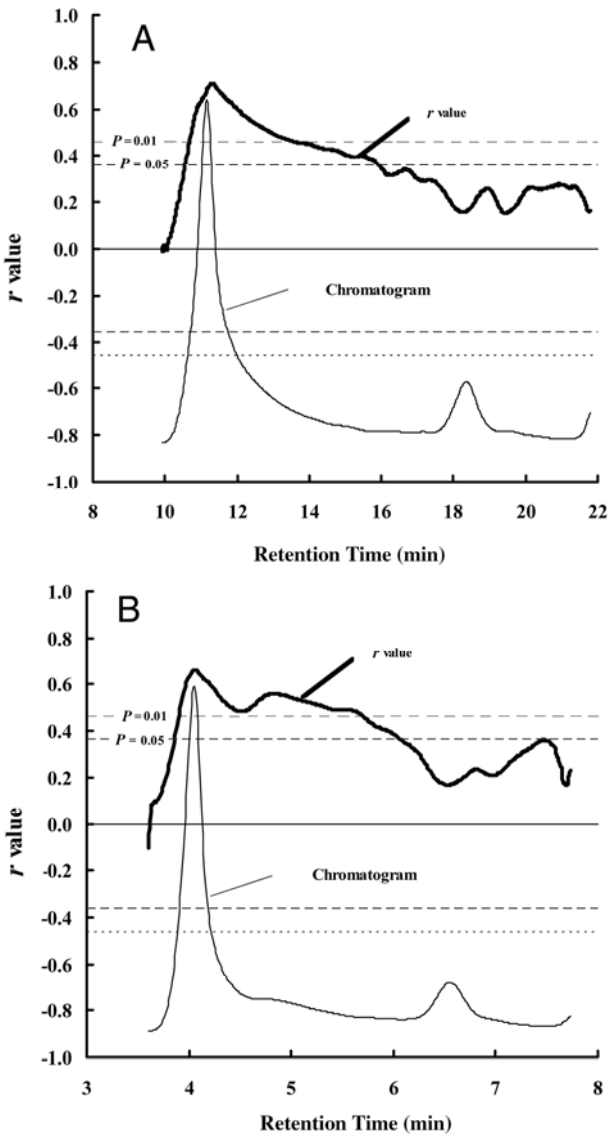

Fig. 5. Correlations (*r)* between farinograph peak time (min) and absorbance area values obtained from size-exclusion HPLC of SDS unextractable proteins using a regular column (**A**) and a narrow bore column (**B**).

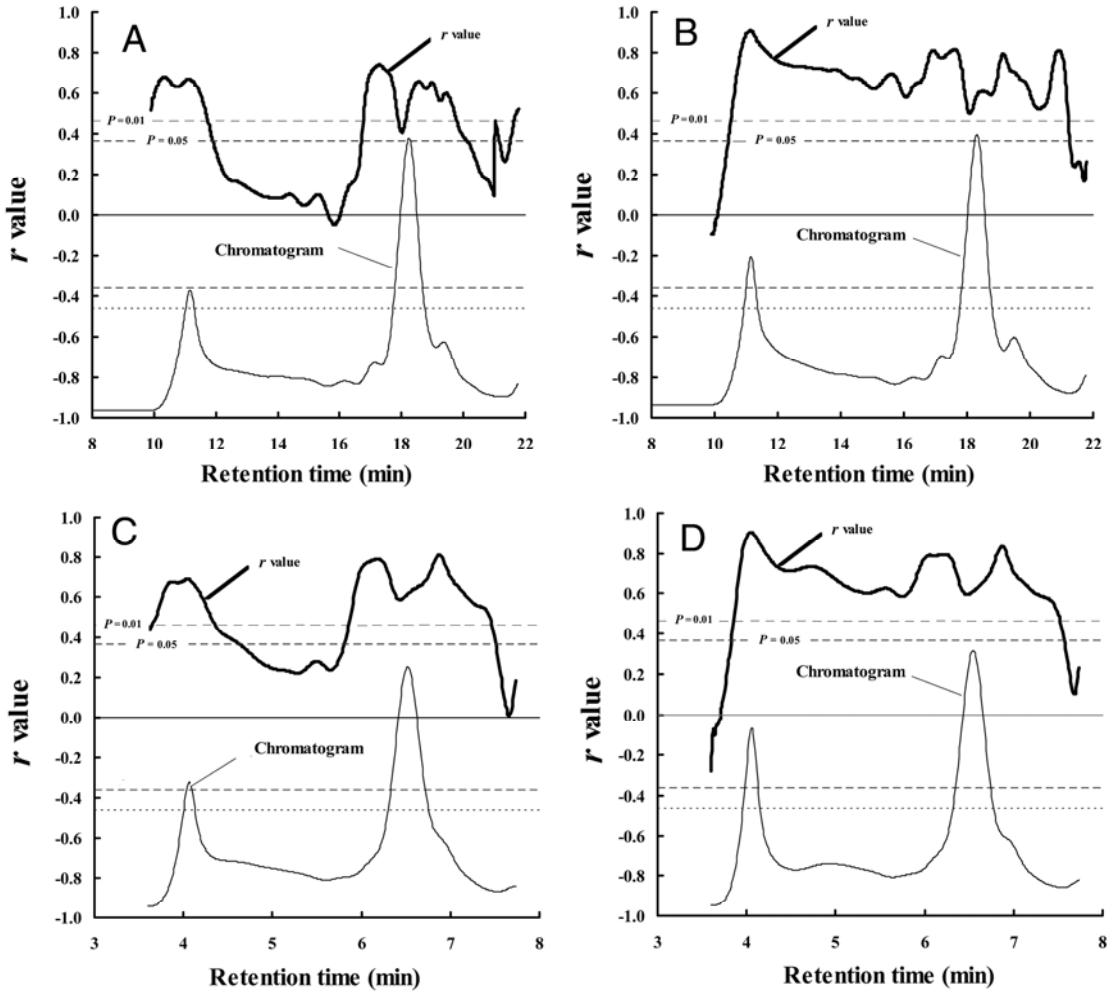

Fig. 6. Correlations (r) between bread loaf volume (cm^3) and absorbance areas of total protein extracts and calculated values obtained from size-exclusion HPLC. Regular column actual extract (**A**) and calculated values (**B**). Narrow bore column actual extract (**C**) and calculated values (**D**).

same distinctive associations for hard and soft winter wheat flour quality characteristics. Depolymerization and repolymerization of protein high molecular weight glutenin subunits occur in the gluten macropolymer during dough mixing and resting stages that greatly affect variation of mixing characteristics and bread loaf volume (Weegels et al 1996a,b; Skerritt et al 1999). Through depolymerization and repolymerization, the interaction of glutenins with other proteins such as gliadins and albumins/globulin could be involved in dough matrix formation as well as interaction of glutenins (Lee et al 2002). The distinct correlations of protein fractions and baking characteristics in this experiment might represent the involvement of those proteins in breadmaking. Specifically, SE-HPLC profiles and correlation spectrum indicated that the NBC could be employed to investigate the effect of those protein fractions on the breadmaking quality of hard spring wheat flours.

CONCLUSIONS

The objective of this experiment was to investigate whether or not an NBC could be employed for analysis of flour protein molecular weight distribution by SE-HPLC and subsequent evaluation of breadmaking quality of hard spring wheat flours. The use of an NBC was also expected to decrease consumption of hazardous organic solvents due to low flow rate with short analysis time. An NBC separated proteins in 10 min, while a regular column took 30 min at a flow rate of 0.5 mL/min. Although the NBC showed a little lower resolution of protein separation, SE-HPLC profiles indicated no pronounced difference between the NBC and the regular column. Flour protein content showed significant ($P < 0.001$) r values with total AA values obtained using the regular column and the NBC, respectively.

Correlation spectrum indicated that AA values from the NBC also had similar r values with mixing and breadmaking parameters to the regular column. Specifically, correlation spectrum indicated that protein fractions had distinctive associations with breadmaking quality characteristics. Ohm et al (2006, 2008, 2009) suggested that multivariate methods could be applied to develop highly predictive calibration models of wheat quality characteristics by combining the distinctive associations of proteins detected by SE-HPLC. The significant correlation profile in this study indicated that SE-HPLC data obtained using an NBC could be also applied for the development of prediction models by multivariate methods.

The AA values of total protein extracts were estimated by adding AA values of extractable and unextractable proteins. Calculated AA values showed larger quantitative variations, higher repeatability, and greater correlations with quality characteristics than those obtained from actual total protein extracts, indicating that the calculated values could be used for flour quality evaluation without an additional extraction of total flour proteins.

In conclusion, the employment of an NBC for SE-HPLC shortens time required for SE-HPLC analysis and consequently contributes to the enhancement of rapid quality evaluation of large sample sets. The improvements also decreased consumption of organic solvents that are hazardous to the environment.

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